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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

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[X] Additional inventors are being named on page 2 attached hereto

TITLE OF THE INVENTION (280 characters max)

IMPROVED PLANTS FOR AGRICULTURAL USE AND METHODS OF USE THEREOF

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Respectfully submitted,

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PROVISIONAL APPLICATION – PAGE 2

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Transgenic cucumber expressing the 54-kDa gene of Cucumber Fruit Mottle Mosaic tobamovirus are highly resistance and protect non-transgenic scions from soil infection

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ABSTRACT

Cucumber fruit mottle mosaic tobamovirus (CFMMV) causes severe mosaic symptoms with yellow mottling on leaves and fruits, and occasionally severe wilting of cucumber plants. No genetic source of resistance against this virus has been identified. The genes coding for the coat protein or the putative 54-kDa replicase were cloned into binary vectors under control of the SVBV promoter. *Agrobacterium*-mediated transformation was performed on cotyledon explants of a parthenocarpic cucumber cultivar with superior competence for transformation. R_1 seedlings were evaluated for resistance to CFMMV infection by lack of symptom expression, back inoculation on an alternative host and ELISA. From a total of 14 replicase-containing R_1 lines, 8 exhibited immunity, while only 3 resistant lines were found among a total of 9 CP-containing lines. Line I44 homozygous for the 54-kDa replicase was selected for further resistance analysis. Line I44 was immune to CFMMV infection by mechanical and graft inoculation, or by root infection following planting in CFMMV-contaminated soil. Additionally, line I44 showed delay of symptom appearance following infection by other cucurbit-infecting tobamoviruses. Infection of line I44 plants with various potyviruses and cucumber mosaic cucumovirus did not break the resistance to CFMMV. The mechanism of resistance of line I44 appears to be RNA-mediated, however the means is apparently different from the gene silencing phenomenon. Homozygote line I44 cucumber as rootstock demonstrated for the first time protection of a non-transformed scion from soil inoculation with a soil borne pathogen, CFMMV.

INTRODUCTION

A new cucurbit-infecting tobamovirus was characterized from greenhouse cucumbers (*Cucumis sativus* L.) in Israel, the cucumber fruit mottle mosaic virus (CFMMV) [1]. Cucumber varieties are susceptible to 5 distinct tobamoviruses separated into two subgroups [1, 10, 11]. CFMMV is closely related biologically and in sequence to the Kyuri green mottle mosaic virus (KGMMV)[1, 30] and Zucchini green mottle mosaic virus (ZGMMV) [23]. However, shared sequence and serological homology with CFMMV and cucumber green mottle mosaic virus CGMMV-W > @are lower. CFMMV is the most damaging tobamovirus on cucumber, and as no stable naturally occurring resistance has yet been found, the only approach for development of resistance is by pathogen-mediated resistance via the production of transgenic cucumber.

Under commercial greenhouse conditions, symptoms of CFMMV are first recognized on fruit and apical leaves at a relatively advanced growth stage. Symptoms on fruit are cultivar-dependent, affected by high temperature and include bright mottling or mosaic. Leaf symptoms include severe mosaic, vein banding and yellow mottling. In some cases, fully developed plants show severe wilting symptoms leading to plant collapse. Rapid viral spread within greenhouses may lead to significant economic losses in cucumber crops. The virus spreads easily via mechanical contact of plant organs with a source of inoculum. Tobamoviruses particles are known to be stable in dead plant tissue in the soil for a long period (Hezi find REF). Virus particles in the soil have a potential to cause a primary epidemic, mainly in greenhouses where many seedlings are densely planted. An efficient virus inoculation of cucumber via soil containing CFMMV in the field was determined, progressing to a devastating disease (Antignus unpublished data).

The CFMMV RNA genome consists of 6,562 nucleotides (nts) (accession no. AF321057) [1] with three subgenomic RNAs coding four ORFs. The 5` proximal region

encodes two coinitiated proteins essential for replication - the 132-kDa and 189-kDa proteins. The 189-kDa protein is created by read-through of the stop codon of the 132-kDa protein by a leaky UAG terminator codon at the 3`-end of 132-kDa at position 3563 (accession no. AF321057). The subgenomic RNA starts from the end of the small replicase gene (132-kDa) (termed I1-RNA) [28], encoding the read-through portion of the replicase gene - the putative 54-kDa protein as yet not identified in plants infected by tobamoviruses [34]. The two other proteins encoded from subgenomic RNAs are a 28-kDa putative MP (774 nts), and the 17-kDa CP (486 nts) [1].

A number of strategies involving pathogen-mediated resistance have been employed to produce virus-resistant plants transformed plants bearing portions of a viral genome (reviewed by: [4, 6, 21]). Resistance may be due to the expression of protein or non-translated RNA. The expression of tobacco mosaic virus (TMV is the type member of tobamovirus) coat protein (CP) in transgenic tobacco was the first demonstration of genetically engineered resistance to a plant virus [22]. In general CP-mediated resistance was effective against a wider range of viral strains, without displaying strong immunity to inoculation as can be shown by RNA-mediated resistance [4, 17]. A high level of resistance has been noted several times in transgenic plants harboring a non-functional replicase segment (reviewed by [7, 21]). Replicase-mediated resistance in most cases is limited to strains sharing high sequence homology, is not affected by virus titer, and is not correlated with the transgene expression level. Transgenic tobacco expressing the 54-kDa gene [14] and a defective 183-kDa gene [9] of TMV show strong resistance to TMV, although with the second (183-kDa) construct a broader resistance was observed than in the first (54-kDa). Additionally, transgenic *N. benthamiana* expressing the intact and truncated 54-kDa protein coding sequence of pepper mild mottle tobamovirus (PMMV) exhibited high levels of resistance to PMMV infection [31]. In contrast it has been shown that expression of the 54-

kDa protein of PEBV [18] in *N. benthamiana* and the 54-kDa protein of TMV in protoplasts [5] was essential for resistance to infection of the homologous viruses.

We report here a transgenic parthenocarpic cucumber highly resistant to CFMMV infection following transformation with the 54-kDa gene. In addition, we show for the first time protection of a non-transformed scion from soil infection with a tobamovirus by grafting onto CFMMV-resistant rootstock.

MATERIAL AND METHODS

Construction of the binary vector pCam54-kDacy and pCamCPcy

The ΔSVBV promoter (accession no. X97304) [33] attached to the 5` non-coding region (NCR) of ZYMV was PCR amplified from the template ΔSVBVpr-ZYMV-FLC clone [33] with the ΔSVBV sense primer (5'C GCT AGC TAT CAC TGA AAA GAC AGC3 ') and the ZYMV NCR antisense primer harboring a N*co*I site underlined (5'GGCCATGGTTATGTCTGAAGTAAACG3 '). The PCR fragment (470 bp) was cloned into the pGEM-T vector (Promega) designated pΔSVBV-NCRzy. The putative 54-kDa gene and the coat protein (CP) of CFMMV (accession no. AF321057) were PCR amplified from clone pUC3'-3.3kb as a template [1] with the following primers: 5'CGGCCATGGCATCGAAGGCGGGTTTTTGGACG3 (54-kDa sense with N*co*I site underlined), 5'GAGGTGACCTAGACACTAGGCTTAATGAATAG3' (54-kDa antisense with B*stE*II site underlined), 5'TGGCCATGGCTTACTCTACTTCTGG-3'(CP sense with N*co*I site underline) and CP antisense primer with B*stE*II 5'CCGGTGACCTCACTTCGAGGTAGACGACGACGC3'. The putative 54-kDa PCR fragment (1461 bp) and the CP PCR fragment (486 bp) were digested by N*co*I/B*stE*II and cloned separately into pΔSVBV-NCRzy digested by N*co*I/B*stE*II . The obtained clones pΔSVBV-NCR54-kDa and pΔSVBV-NCRCP were double digested by *EcoR*I/B*stE*II and the resulted insert was cloned into the binary vector pCAMBIA2301[15] which was double digested with *EcoR*I/B*stE*II prior to cloning. The new binary vector pCAMSV54-kDa and pCAMSVCP (which does not contain the ß-glucuronidase gene) were digested by *EcoR*I /B*gl*II in order to replace the 35S promoter located upstream of the kanamycin gene with the intact SVBV promoter. The SVBV promoter, previously cloned into pGEM-T and

termed SVBVpr [33], was removed by double digestion with *EcoRI*/*Bgl*II sites and cloned into the same sites in the new binary vectors pCAMSV54-kDa and pCAMSVCP. These two final constructs were used for cucumber transformation (Fig. 1).

Cucumber transformation. Aaron please complete this paragraph The binary plasmids bas on Cambia binary vector: pCAMSV54-kDa and pCAMSVCP, harboring the putative 54-kDa gene and coat protein (CP) were transformed to *Agrobacterium tumefaciens* XXX strain.

Cucumber (*Cucumis sativus* L. breeding line I) seedlings were transformed as follows....

Selfed progeny (R1 seeds) from fertile plants were germinated on aseptic selective medium, in order to verify the expression of kanamycin resistance and to screen for homozygous lines. Susceptibility to kanamycin (100 µg/ml) was manifested in young seedlings as a severe inhibition of elongation of the primary root and lack of root branching.

Plants, viruses and inoculation. Cucumber (*Cucumis sativus* L. cv. XXXX) plants were grown in a temperature-controlled greenhouse at 25°C with a photoperiod of 14 hr and in controlled growth chamber at 20-35°C. Cucumber seedlings were used as source plants to maintain viral cultures for inoculation of CFMMV [1], KGMMV [30], ZGMMV [23], CGMMV [11] and cucumber vein yellowing virus CVYV [16]. Squash plants were used as source of inoculum of zucchini yellow mosaic virus ZYMV [13] and cucumber mosaic virus CMV Fny strain [12]. Inoculations of cucumber plants were conducted mechanically by rubbing cotyledons (3 days post germination) after dusting with carborundum. Inocula were extracted by grinding young leaves of source plants in distilled water.

Evaluation of the resistance response. Kanamycin-resistant R1 seedlings from putative transgenic progenies were screened under greenhouse conditions for viral resistance, by mechanical inoculation with purified CFMMV at 1 mg/ml in 50 mM phosphate buffer (pH 7.4) or RNA at a concentration of 400 µg/ml in 50 mM phosphate buffer (pH 8.0). For most transgenic progeny more than 10 seedlings were initially screened by inoculation. Inoculated seedlings were kept for several weeks under greenhouse conditions. The percentage of infection and/or resistance of non-transformed or transformed plants were determined by visual inspection and by ELISA in all experiments [1].

ELISA assays. Inoculated cucumber lines were analyzed for the presence of CFMMV by DAS-ELISA using a specific antiserum prepared against CFMMV in 1:1,000 dilution [1].

Soil and graft inoculation: FOR HEZI

Virus purification: Purified virion of CFMMV for inoculation of plants was extracted by ...FOR HEZI.....

(Hezi this is the protocol which used by Ryu) For evaluation of resistance the tobamoviruses KGMMV, ZGMMV, CGMMV and CFMMV were partially purified from infected cucumber according to > @with some modifications. Systemic infected leaves (1.3 g) were ground in 0.1 M phosphate buffer (pH 7.0), and precipitated following addition of butanol to 0.5% and PEG to a final concentration of 4%. The partially purified precipitant virus pellet preparation was analysed by protein gel and western blotting.

Grafting of non-transformed scions on transformed rootstocks. Cucumber seedlings at the cotyledon stage were used for grafting. A top grafting method was employed in which the non-transformed scion was grafted following a diagonal cut of the stem under the cotyledonary node and installed on top of a transgenic or non-transformed rootstock line, which was also cut diagonally at the cotyledonary node in a manner that retained a single cotyledon. The scion/rootstock junction was secured in place with small plastic clips. During the first week grafted plants were maintained in a high humidity.

Extraction and analysis of RNA. Expression of the viral transgene RNA was tested by northern blot and RT-PCR analysis of total RNA extracted from seedlings three weeks after germination. Young leaf tissue (300 mg) was ground to a fine powder in liquid nitrogen and RNA was extracted by TRI-REAGENT kit (Molecular Research Center, Inc., Cincinnati, OH, USA), according to the manufacturer's instructions. RNAs concentrations were measured by GeneQuant (Pharmacia Biotech), and comparable amounts of RNAs were loaded on the gel. About 30 μg of each sample were electrophoresed in a denaturing 1.5% agarose gel containing formaldehyde. The separated RNAs in the gels were then blotted onto Hybond-NX membranes (Amersham, Piscataway, NJ, USA) and fixed by exposure to UV for 2 min. Prehybridization with Rapid-hyb buffer (Amersham) was performed for 2 hours. Transgene RNA and plus-sense CFMMV RNAs were detected by hybridization with ^{32}P-labeled cDNA probe of the putative 54-kDa gene of CFMMV of positions 3824-4693 (869 bp) (accession no. AF321057) with a random primed ^{32}P-labeled DNA probe (Random primer DNA labelling mix kit; Biological Industries, Kibbutz Beit HaEmek, Israel). RT-PCR for detection of CFMMV infectivity was conducted in a one-tube single-step method

with 2-5 µg total RNA according to Arazi et al. [2] with specific primers of the coat protein (sense 5' GAGGTGACCTAGACA CTAGGCTTAATGAATAG3 ' and antisense 5' CCG GTGACCTCACTTCGA GGTAGACGACGACGA3 ') and the 54-kDa gene (sense 5' GCT ACG GAG CGT CCG CGG 3 ' and antisense 5' CGC GGT CGA CTG TAT GTC AT3 '). RT-PCR cycles were as follows: 46°C 30 min; 94°C 2 min, followed by 5 cycles at 33 cycles at 94°C, 58°C and 72°C, each of 1 min, followed by 35 cycles at 94°C, 58°C and 72°C, each of 30 s., and one final cycle of 5 min at 72°C. RT-PCR for infectivity assay of various tobamoviruses (Fig. 4) were performed in two steps with specific primers of the CP genome of CGMMV (5'TCTGACCAGACTACCGA AAA3' and 5'ATGGCTTACAATCCGATCAC3') KGMMV (5'GAGAGGATCC ATGTTTCTAAGTCAGGTCCT3' and 5'GAGAGA ATTCTCACTTTGAGGAAG TAGCGCT 3'), ZGMMV (5'TCTATCGCTTAACGC AGC3' and 5'ATGTCTTAC TCTACTTCTGG3') and CFMMV (5'CAAGACGAGG TAGACGAAC 3' and 5'ATGCCTTACTCTACCAGCG3 '). RT-PCR was performed by RT-PCR AmpTaq kit (Perkin Elmer) in an i-cycler (Bio-Rad) and cycling step was 37°C for 1 hr and 30 cycles of 1 min at 94°C, 40 sec at 53°C (KGMMV), 52°C (ZGMMV, CGMMV) and 44°C (CFMMV) the annealing temperature, and 1 min at 72°C, and finally 72°C for10 min.

DNA extraction and PCR Analysis. Total genomic DNA was extracted from young leaves (3 weeks after germination) of each individual R_1 line and homozygotic line I44 by CTAB methods (REF). DNA solution (1 µl) was diluted in 25 µl PCR reaction mixture, containing primers according to the sequence of CFMMV 54-kDa gene (accession no. AF321057). Two sets of primers were used. The first primer set at positions 3824 and 4693 (5' GCTACGGAGCGTCCGCGG3` and 5'CGCGGTCGACTGTATGTCAT3') (see results Fig. 3) and a second primer set at positions 3785 and 4479 (5'GAAAAAGGAGTTTTTGATCCCGCT3' and 5'ACTGATATG CGTCTTCTTATGCCC3'). PCR conditions were: one cycle of 2 min at 94°C and 35 cycles at 94°C, 58°C and 72°C, each of 30 sec, and finally 5 min at 72°C.

Protein analysis by western blotting. Partially purified virions were mixed 1:1 with 2X SDS-PAGE loading buffer and boiled for 5 min [24]. The boiled material (10 µl) was fractionated by SDS-PAGE on a 12.5% polyacrylamide gel. The fractionated proteins were electroblotted onto nitrocellulose membranes (Amersham), blocked with non-fat dry milk, and probed with a mixture of rabbit antisera specific against CGMMV capsid protein (1:1,000 dilution) and ZGMMV capsid protein (1:2,000 dilution). The membranes were

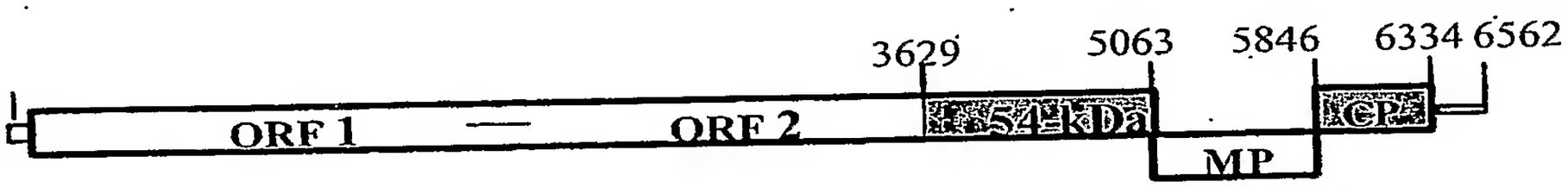
then incubated with goat anti-rabbit IgG conjugated to alkaline phosphatase (1:4,000 dilution) and the viral capsid proteins were visualized by incubating the membranes with the chromophore substrates NBT and BCIP (Promega, Wisconsin, USA) as described by [24].

RESULTS

Construction of binary vectors with CFMMV coat protein gene or the putative 54-kDa replicase gene.

The intact CP of CFMMV (488 bases) and the putative 54-kDa encoded gene with addition of 22 nt upstream of the Met position 3629 (1456 bases from nucleotides 3607 to 5063) [1] (Fig. 1A) were cloned separately into the pCambia2301 binary vector (accession no. AF234316) [15]. The CP and putative 54-kDa genes of CFMMV were fused to the non-coding region (NCR) of ZYMV at the 5'-end and to the Nos poly-A terminator at the 3'-end. The target genes (CP and 54-kDa) and the NPTII gene for selection on kanamycin were cloned adjacent to the truncated and the full length SVBV promoters respectively [33] Fig. 1B.

A.



B.

Fig 1. Schematic presentation of the genome organization of cucumber fruit mottle mosaic tobamo virus (CFMMV). The numbers above refer to the position of the genes. The putative 54-kDa and coat protein (CP) genes are marked (gray boxes). (B). the construct for plant transformation between the left (LT) and the right (RT) borders (black). The putative 54-kDa or the coat protein (CP) genes of CFMMV were fused to the ZYMV 5' non-coding region (NCR) between the truncated SVBV (SV) promoter and the Nos poly-A terminator (T). The selective NPTII gene was inserted under the full length SVBV promoter.

R_1 progeny plants were screened for the presence of the putative 54-kDa or CP transgenes by PCR analysis. The insertion of CFMMV genes within the plant genome was confirmed for all of the kanamycin-resistant R_1 lines indicated in Table 1. PCR fragments of 1456bp and 488bp were detected using specific flanking primers for the 54-kDa and the CP respectively (data not shown). In resistance screening following CFMMV infection with 1mg/ml of purified virus, eight of fourteen of replicase-encoding R_1 lines and three of nine of CP-containing lines exhibited full resistance ('immunity") (Table 1). Otherwise, both

fully susceptible lines, and partially resistant lines such as R149, R181 and CP238 were observed. Interestingly, in contrast to the "immune" putative 54-kDa encoding lines, two symptomless resistant CP lines (CP86 and CP202) trace amounts of virus could be detected, but only by back inoculation on *N. benthamiana* (Table 1 , and data not shown). However, neither symptoms nor accumulation of virions could be observed by ELISA in three lines: CP86, CP112, CP202 (Table 1) following CFMMV inoculation with purified virus and viral RNA. Interestingly, resistance breaking was observed only with line CP86 when the temperature was raised to 30 ^{0}C all day for three weeks. Line CP86 is exceptional (Table 1), since it contained more then one copy of the CP transgene as evaluated by segregation of the R_1 on Kanamycin (data not shown). In addition, expression of the transgene CP protein could not detected in those lines by ELISA.

However the 54-kDa R_1 transformed lines divided into resistant and susceptible groups only, and no biological differences were observed between the 8 resistant lines.

Table 1: Screening of R1 transgenic cucumber lines containing the putative 54-kDa or CP genes for resistance to CFMMV.

Original line~	Response to viral inoculation^	Response to back inoculation#
R44*	**0/64**	
R45*	**0/7**	
R84*	**0/5**	
R187*	**0/3**	**n.t**
R169*	**0/9**	
R175*	**0/17**	
R179*	**0/10**	
R205*	**0/8**	
R149	2/5	
R170	9/9	
R181	7/10	
R28	9/9	
R189	9/12	
R146	6/6	
CP86	**0/25**	
CP112	**0/18**	-

CP202	**0/8**	
CP197	4/9	
CP201	5/5	
CP195	6/6	
CP228	8/8	
CP235	8/8	
CP238	2/8	
Non-transformed cucumber	50/50	+

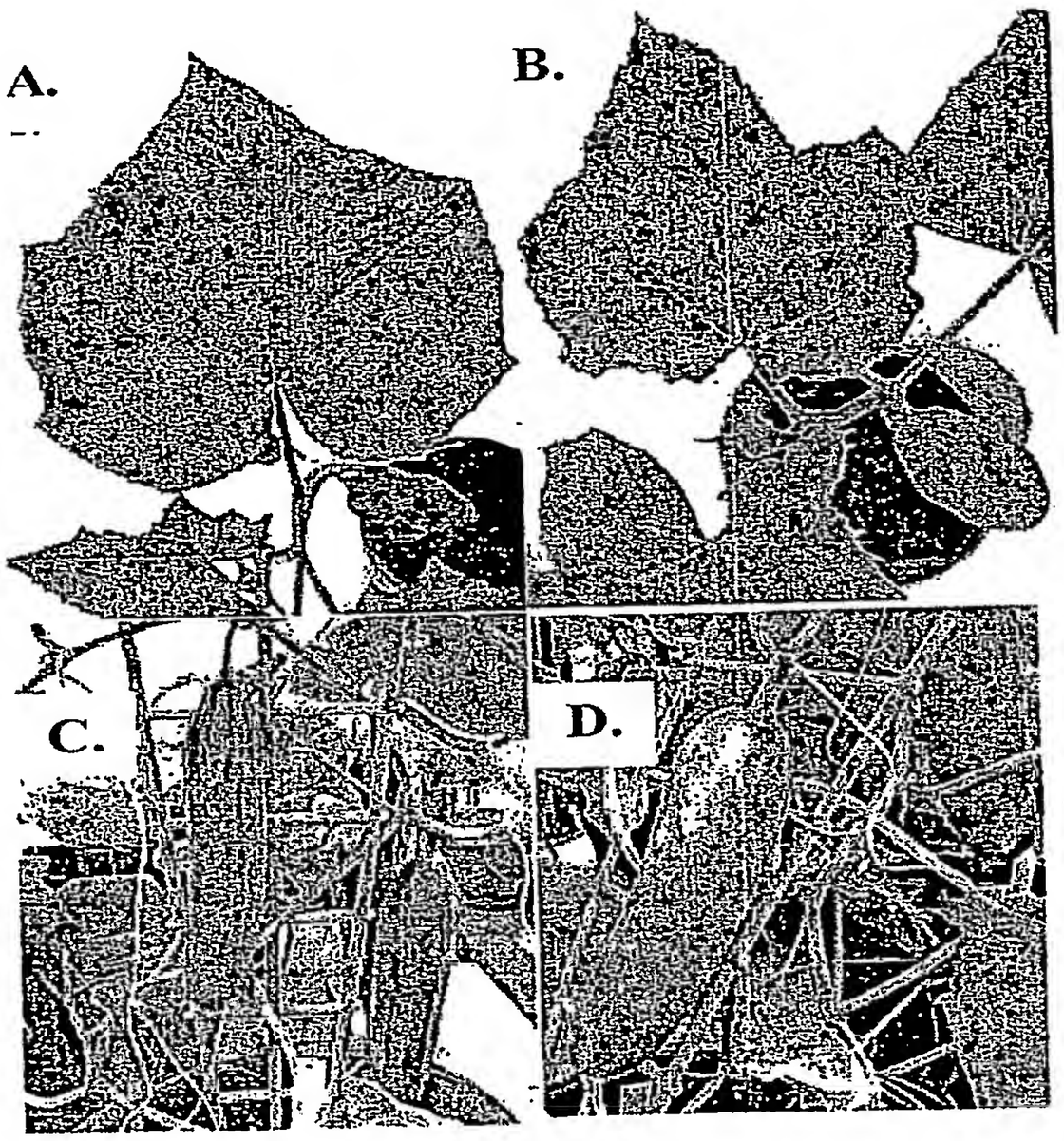
" Each numbered line represents the progeny of individual regenerant R_0 plants which were selfed. and R_1 seeds were germinated in the presence of kanamycin. R_1 lines marked with R or CP refer to constructs contain the putative 54-kDa gene (replicase) or the coat protein (CP) gene.

^ Kanamycin-resistant seedlings were evaluated for resistance to CFMMV by mechanical inoculation with 1mg/ml of purified virus. The number of susceptible seedlings of the total inoculated is shown. Fully resistant ("immune") lines are indicated in bold.

CFMMV-resistant seedlings were evaluated for low levels of CFMMV accumulation by back inoculation of *N. benthamiana*. Systemic symptoms (+) and no symptoms (-) on *N. benthamiana* were observed three weeks post inoculation. n.t. – not tested

Further extensive studies of resistance of transgenic cucumber to CFMMV infection were performed only for the fully resistance (immune) line R44 (see Table 1), now designated as line I44. Line I44 contained a single copy of insert DNA according the segregation of R1 plant on kanamycin XX resistant out of XXX. To eliminate genetic variation, a homozygotic line I44 was selected and used for virus resistance and molecular analysis. Line I44 homozygotic plants were not distinguishable from the normal non-transformed parental line in any horticultural parameter (data not shown). However, in contrast, to the resistant line I44 the parental line was susceptible to mechanical inoculation with CFMMV (Fig. 2). At temperatures above 25 ^{0}C strong mosaic symptoms appear on the parental non-transformed line after 14 days, which progress to deform leaves, stunt plants and cause abnormal fruit with yellow patches and occasionally cracked strips (Fig. 2).

Fig 2: Response of transgenic line I44 (A. and C) and non-transformed (parental line) (B. and D) cucumber to CFMMV inoculation. Symptoms documented from leaves and fruit three and seven weeks respectively after inoculation.



CFMMV is a tobamovirus infecting plants in the field by mechanical inoculation via contact with a source of inoculum on the leaves or stem and by soil inoculation. For this reason we tested once again the resistant line I44 by mechanical sap inoculation from infected leaves and by planting line I44 seedlings in soil contain inoculum. In addition, for an even more aggressive inoculation method, seedlings of line I44 were grafted onto an infected cucumber rootstock. By all of the three different inoculation methods, the homozygotic line I44 remained symptomless, and virus could not detected by ELISA or by back-inoculation on susceptible hosts (*N. benthamiana* and cucumber) (Table 2.)

Table 2. Resistance of line I44 to CFMMV infected by mechanical, soil and graft inoculation.

Inoculation methods	Lines	Infectivity*	ELISA	# Back inoculation
Mechanical inoculation	I44	0/65	-	-
	Control	30/30	++++	++++
Soil inoculation	I44	0/10	-	-
	Control	8/10	++++	N.T
Graft inoculation	I44	0/16	-	-
	Control	12/16	++++	N.T

*Infectivity rate of transformed (I44) and non-transformed (parental control) plants were scored as number of infected plants from the total number of plants inoculated, by visual symptoms 4 weeks post inoculation.

Back inoculation was evaluated by mechanical inoculation of *N. benthamiana* with sap extracted from inoculated cucumber 4 weeks post inoculation.

NT = not tested

The presence of the transgene DNA in the line I44 genome was verified by PCR from the total DNA extraction (Fig. 3 panel C, DNA-ex). In addition expression of RNA transcript of the putative 54-kDa transgene was detected by RT-PCR only in line I44 plants (Fig.3 panel A). However, PCR reaction from the RNA extraction was negative, indicating that the expression of transgene RNA is not due to residues of DNA in RNA extraction (Fig.3 panel C). To verify that virus did not accumulate in line I44 following inoculation with CFMMV (I44 inoc.), in addition to visual symptoms an RT-PCR was performed (Fig.

3B). No amplified band of CFMMV was obtained by RT-PCR with specific primers of CFMMV CP RNA in I44 line in contrast with an positive CP band (486 bp) in non-transformed infected plants (Fig. 3B). These results indicate once again that no virus accumulated in I44 plants.

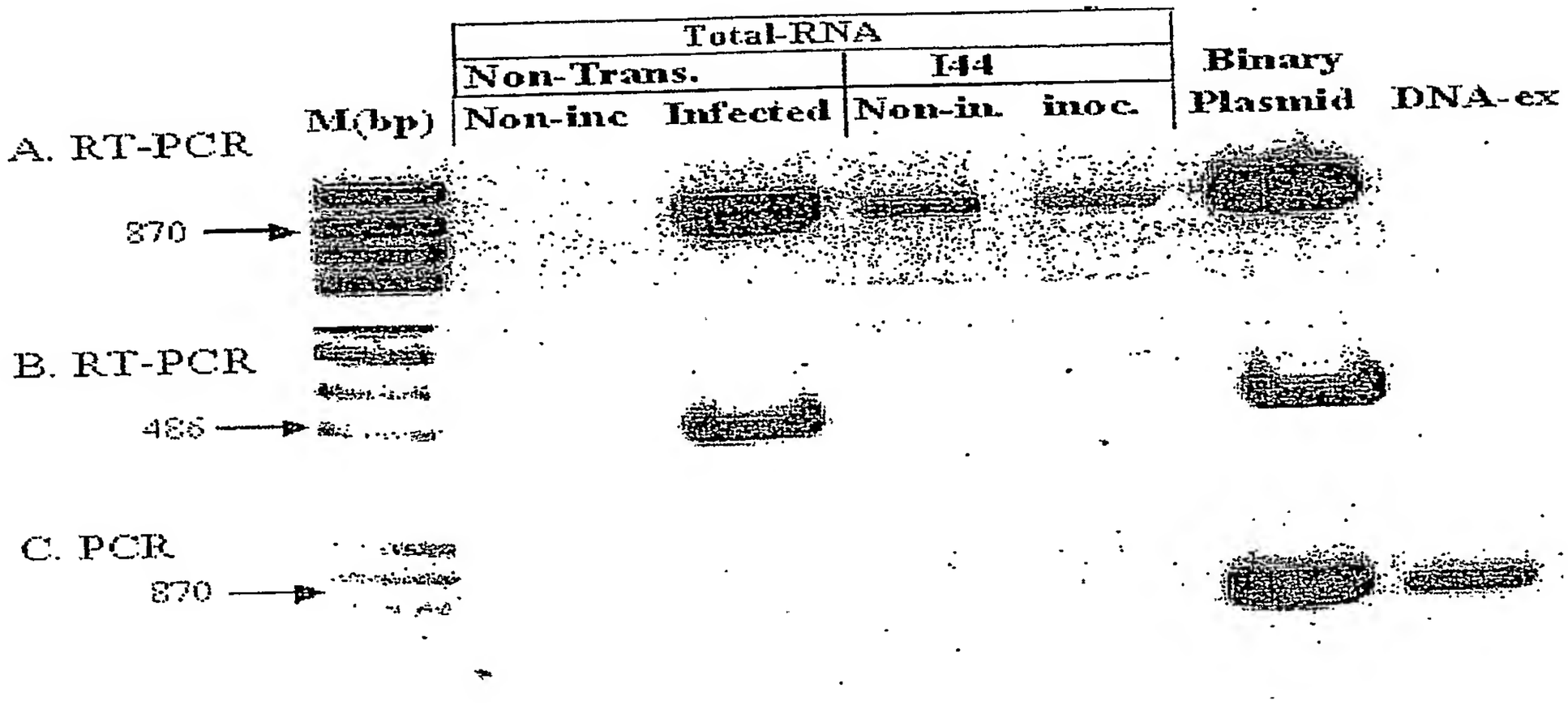


Fig 3. Verification of the resistance of line I44 to CFMMV infection by RT-PCR analysis. Total RNA extracted from transgenic line (I44) and non-transformed (control) plants three weeks post inoculation with CFMMV. Total RNAs extracted from inoculated and non-inoculated plants (transformed and non-transformed) serve as a template for RT-PCR (A and B) and PCR (C) analysis. Detection of transgene DNA within line I44 genome was determined by PCR from DNA extraction (DNA-ex) panel (C).

Replicase mediated resistance is known to be sequence specific [20], and therefore it was important to examine the resistance of the transgenic line I44 against infection with different cucurbit-infecting tobamoviruses. The transgenic homozygotic line I44 and non-transformed plants were inoculated by three additional tobamovirus; KGMMV and ZGMMV which share high sequence homology with CFMMV, and CGMMV which has a low sequence homology within the CP gene with CFMMV [1]. The resistance was

examined by visual symptoms at differences time post inoculation (Table 3) and with virus accumulation by RT-PCR and western blot (Fig. 4 A, B). The non-transformed (parental line) shows symptoms 8 days post inoculation (dpi) with KGMMV and ZGMMV and two days later symptoms appear with CFMMV and CGMMV (Table 3.). However, with the homozygotic line I44, symptoms become visible 14 dpi with CGMMV and ZGMMV, and 20 dpi with KGMMV. In addition, milder symptoms were observed for CGMMV infection of I44 plants during the whole period up to 30 dpi, in contrast to the severe symptoms in non-transformed plants observed 14 dpi. At 30 dpi line I44 plants shows resistance only against CFMMV infection by suppression of symptom expression (Table 3).

Table 3. Resistance of the I44 transgenic plants to infection with different cucurbits tobamovirus, CGMMV, KGMMV, ZGMMV and CFMMV:.

Date (dpi)	**CFMMV-54-kDa transgenic cucumber (I44)**				**Non-transgenic cucumber**			
	CFMMV	CGMMV	KGMMV	ZGMMV	CFMMV	CGMMV	KGMMV	ZGMMV
8	-	-	-	-	-	-	++++	++++
10	-	-	-	-	+++	+++	+++++	+++++
14	-	+	-	+++	++++	++++	+++++	+++++
18	-	+	-	++++	+++++	+++++	+++++	+++++
20	-	+	++	++++	+++++	+++++	+++++	+++++
24	-	+	++++	+++++	+++++	+++++	+++++	+++++
26	-	+	+++++	+++++	+++++	+++++	+++++	+++++
30	-	+	+++++	+++++	+++++	+++++	+++++	+++++

Cucumber plants were inoculated at the cotyledon stage and symptoms (+) were determined for resistance (-) every four days. The severity of symptoms is sum of mosaic spread and stunting, were scored from 1+ to 5+ for the period of 30 days post inoculation (dpi). The data is summarized from two independent experiments with ten plants for each treatment.

The resistance and susceptibility of line I44 to tobamovirus infection (Table 3.) were additionally confirmed by RT-PCR and protein analysis (Fig. 4 A and B). Viral RNA of CGMMV, KGMMV and ZGMMV were detected in transgenic line I44 plants and the in non-transformed control plants by RT-PCR analysis (Fig. 4). In contrast, CFMMV RNA was not detected in line I44 plants by RT-PCR, but was clearly found in the non-

transformed plants. The transgenic homozygotic line I44 was confirmed by the transgene putative 54-kDa DNA insert (870bp) obtained with PCR analysis (Fig. 4.A upper arrow). In addition, accumulations of the tobamoviruses in non-transformed and line I44 plants was determined by purification of virions 40 dpi (Fig. 4B). The viruses ZGMMV, CGMMV and KGMMV accumulated in both transgenic and non-transgenic plants; however CFMMV virions were extracted only from non-transformed control plants (Fig. 4B).

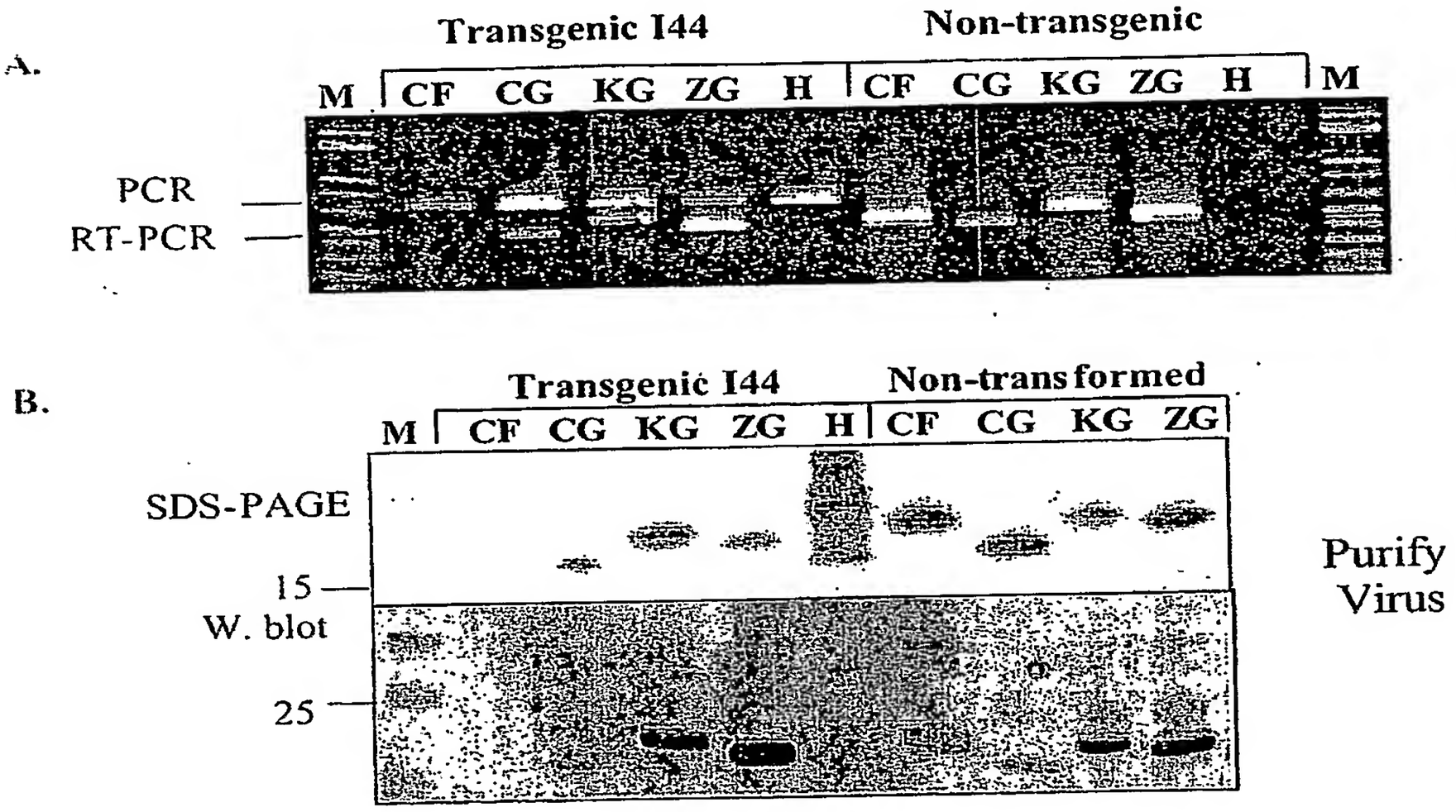


Fig. 4. Evaluation of line I44 resistant plants to infection with different cucurbit-infecting tobamoviruses. CFMMV (CF), CGMMV (CG), KGMMV (KG) and ZGMMV (ZG) by RT-PCR (A). Virion accumulation (B upper panel) and western blot (B lower panel) analysis. Transgenic and non-transgenic plants were distinguished by PCR (indicated by the upper arrow in A) with putative 54-kDa gene specific primers. RT-PCR was performed three weeks post inoculation with specific primers for each virus (indicated by the lower arrow in A). Samples from PCR and RT-PCR reactions from each treatment were loaded in the same lane and separated on 1% agarose gel (A). Forty dpi the viruses were partially purified from the inoculated line I44 and non-transformed control plants and separated on 12% polyacrylamide gel stained by coomassie brilliant blue (B upper). The coat proteins of the partial purified viruses were blotted onto a nitrocellulose membrane and detected using a

mixture of specific anti-ZGMMV and anti-CGMMV antibodies. Lanes: M = molecular weight marker (kDa); and H = mock inoculated plants.

Transgenic line I44 plants show immunity only to CFMMV infection, although a delay with of symptom appearance is observed following infection with other tobamoviruses. Such specific resistance could be explained by RNA-mediated resistance, possibly associated with RNA silencing. In order to characterize the mechanism of resistance of homozygotic line I44, RNA expression studies were made with additional biological tests.

Total RNAs were extracted from transformed line I44 and non-transformed (parental genotype) plants before and after inoculation with CFMMV (Fig. 5). An equivalent amount of total RNAs in each sample were subjected to northern blot hybridization, as determined by the UV intensity of rRNA on the membrane (data not shown). Northern blot hybridization was performed of non-transformed and line I44 plants inoculated with CFMMV, using the labeled putative 54-kDa gene as a probe. The probe hybridized with CFMMV genomic RNA (upper band), 54-kDa subgenomic RNA I1 (Sulzinski et al., 1985) and with the transgene putative 54-kDa transcripts (Fig. 5. NTcf). As expected the transgene putative 54-kDa transcript in I44 plants is shorter than the 54-kDa subgenomic RNAI1 observed in the infected non-transformed control plants (Fig. 5). Expression of transgene putative 54-kDa transcripts was observed for the transformed line I44 plants, but not for the non-transformed (NT) control plants. In addition, inoculation of resistant line I44 plants with CFMMV (cf) and ZYMV (zy) did not affect the expression level of the 54-kDa transgene transcripts (Fig. 5.).

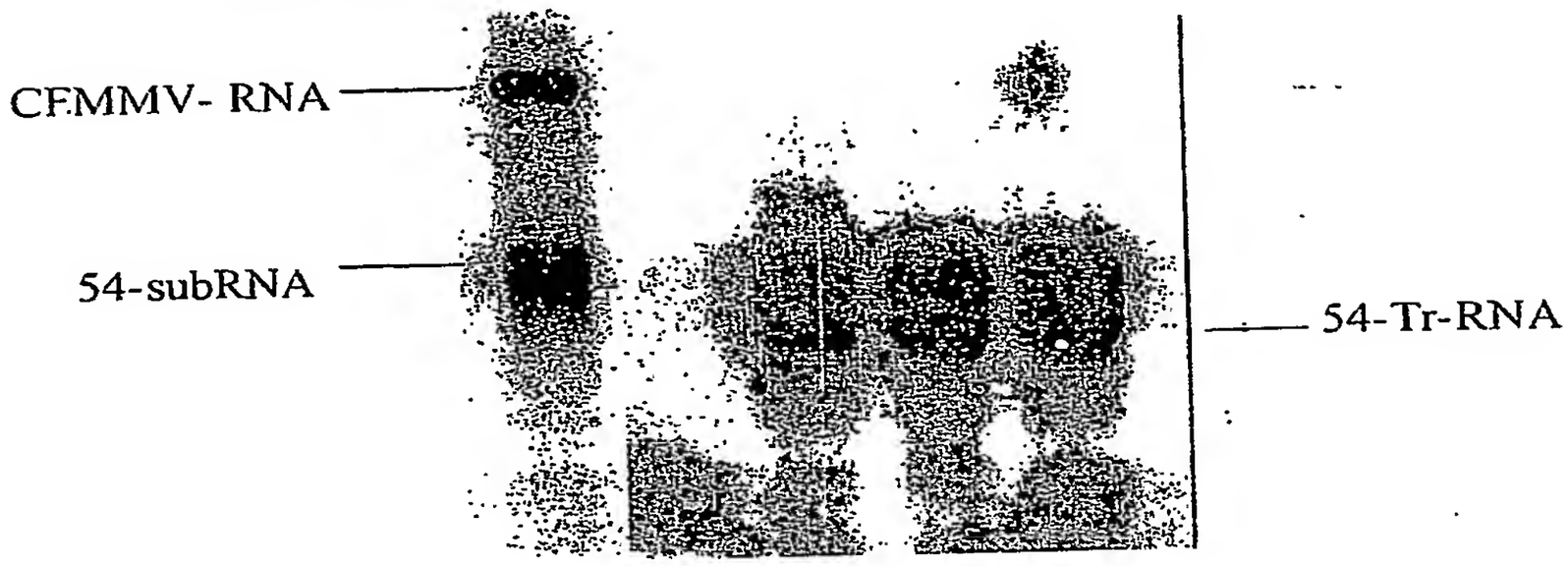


Fig. 5. Expression of transgene RNA in transformed cucumber plants (I44). Total RNAs were extracted from the second and third true leaves of transformed (I44) and non-transformed plants (NT) 18 dpi with CFMMV (cf) or with ZYMV (I44z). RNAs were analyzed by denaturing agarose gel electrophoresis and northern blot hybridization. About 30 µg of RNA were loaded per lane from the transgenic plants. and 3 µg of RNA were loaded from non-transformed control plant infected with CFMMV (NTcf). ^{32}P-labeled RNA probes used for detecting transgene RNAs were complementary to the putative 54-kDa gene. The electrophoretic positions of CFMMV RNA and 54-kDa RNA are indicated adjacent to the panels. Line NTcf (non-transformed plants infected with CFMMV) were exposed for 4 hours while the others were exposed for 3 days.

It has been shown that the mechanism of RNA-mediated resistance by silencing could be broken following inoculation with a potyvirus which suppress the silencing [25], or by elevating the temperature [29]. Plants of line I44 were evaluated for resistance to CFMMV following inoculation with three potyviruses: ZYMV, zucchini fleck mosaic virus (ZFMV) and cucumber vein yellowing virus (CVYV) (of the genus Ipomovirus; family Potyviridae) and with cucumber mosaic virus CMV (Table 4). The transgenic line I44 plants remained resistant to CFMMV in all the mixed infections, while the non-transformed plants showed very severe symptoms in the mixed infection (Table 4) and in the presence of CFMMV, as confirmed by ELISA (data not shown).
In addition, temperature breaking resistant was not observed in line I44 plants inoculated with CFMMV under growth conditions of 20, 28 and 33 ^{0}C (data not shown).

Table 4. Evaluation of homozygotic transgenic line I44 for resistance to CFMMV infection following inoculation with viruses belong to potyviridae family and cucumber mosaic virus (CMV).

Line	ZYMV+ CFMMV	ZYFV+ CFMMV	CVYV+ CFMMV	CMV+ CFMMV	CFMMV+ CFMMV
I44	0/6	0/6	0/6	0/6	0/6
Non-transformed	4/4	6/6	6/6	4/4	6/6

*Infectivity rate of transformed (I44) and non-transformed (parental control) plants were determined as number of infected plants from total number of plants inoculated, as determined by visual symptoms 4 weeks post infection.
#Transgenic homozygotic line I44 and non-transformed cucumber seedling were inoculated with the potyviruses ZYMV-JV, zucchini yellow fleck potyvirus (ZYFV), cucumber vein yellowing virus (CVYV) and CMVa week after germination. Ten days post inoculation the plants were challenge inoculated with CFMMV.

It has been known for many years that tobamovirus particles are very stable in soil and become a source of inoculum for newly planted seedlings (REF). Infection by CFMMV is very common in the greenhouse or nethouse and primary infection by soil contamination could explain the epidemic in protected cultivation

Grafting of cucurbits is a common commercial practice, where the rootstock carry a new trait such as resistance to soil pathogen (REF). The transgenic line I44 showed resistant to soil inoculation of CFMMV (Table 1) and therefore was tested as rootstock to prevent soil inoculation of CFMMV to non-transformed scion. Non-transformed cucumber plants were grafted on homozygotic line I44 (Fig. 6A) and planted in soil containing CFMMV (Fig. 6B). None of the scions became infected with CFMMV at 5 weeks post planting, as verified by visual symptoms, ELISA test and back inoculation on *N. benthamiana*. However in controls where seedlings grafted on non-transform rootstock, 12 out of 16 plants showed visible symptoms of CFMMV, confirmed by ELISA (Table 7).

Table 7. Protection of non-transform scion seedling from soil infection by grafting on transgenic CFMMV-resistant line I44.

	Symptoms of CFMMV on scion	**ELISA#**	**Back # inoculation**
Grafted on I44	0/16	—	—
Not grafted	12/16	+++	**n.t**

* Infectivity rate of non-transformed plants grafted or not grafted on I44, were determined as number of infected plants from total number of plants that planted in soil contained CFMMV inoculum, as determined by visual symptoms 4 weeks post infection.

ELISA and Back inoculation of *N. benthamiana* with sap extracted from scion 4 weeks post soil inoculation were evaluated for infectivity. NT = not tested

A.

B.

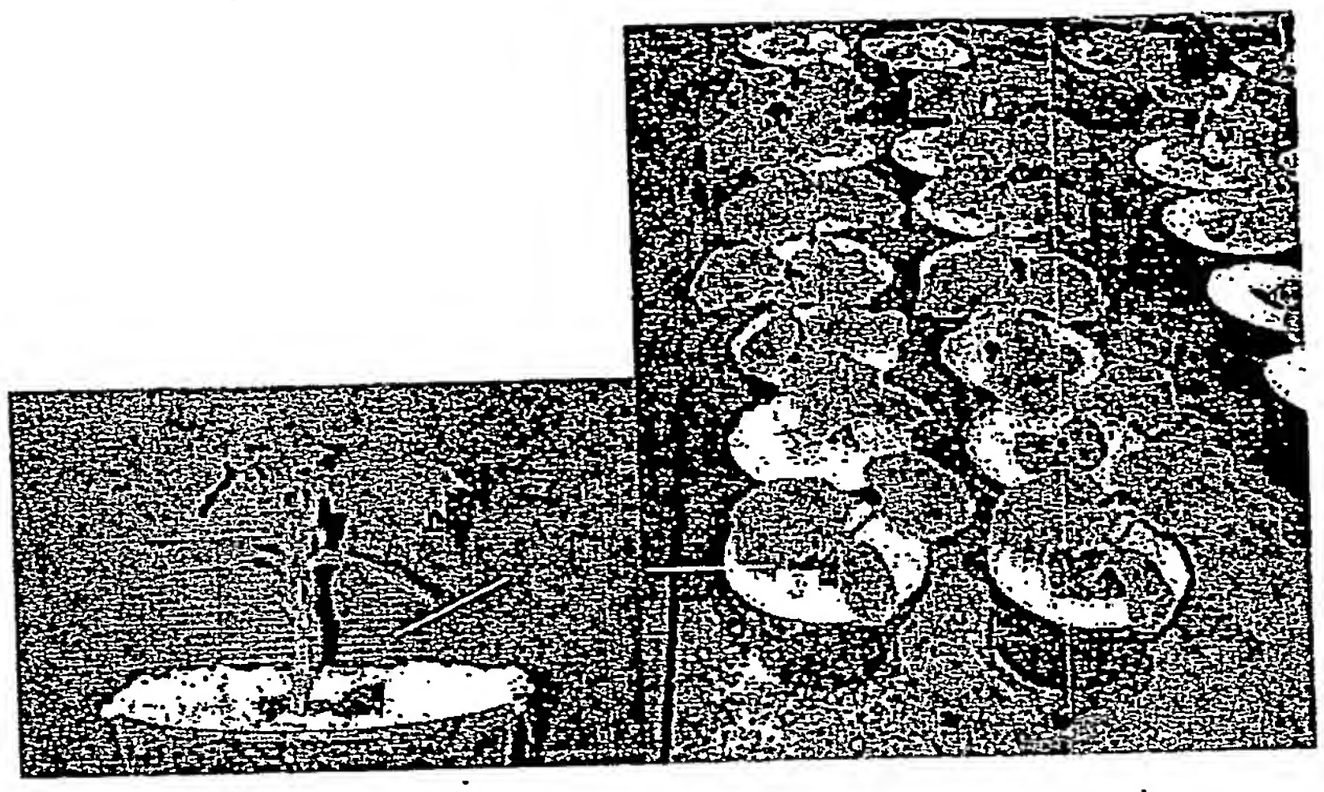


Fig. 6. Grafted cucumber seedling with transgenic line I44 as a rootstock and non-transformed parental line as a scion. Top grafting was made with the seedlings at the coiyledon stage, while the lower non-transform. Grafted seedlings were planted in soil contained CFMMV virus (B).

DISCUSSION:

Parthenocarpic cucumber transformed with the intact structural CP gene and the putative non-structural 54-kDa gene of CFMMV were evaluated for resistance to CFMMV infection. The CP transformed lines were found to be less efficient in CFMMV resistance (3 lines resistant out of 9) than the 54-kDa (8 lines of 14 resistant). In addition, two of the resistant lines (CP86 and CP202) retained a low level of virus after inoculation, as determined by back-inoculation.Additionally, the resistance of line CP86 was broken at high temperature (30 °C). Breakage of resistance at high temperature could be due to aggressive effect (severe symptoms and high titer) of CFMMV at higher rather than at lower temperature (Antignus unpublished data) thereby overcoming the resistance. Alternatively, resistance breakage could be due to the copy number of line CP86, which is higher than the two other resistant lines.

CP resistant lines (CP86, CP202, CP112) did not express detectable levels of CFMMV CP as determined by ELISA, and remained resistant to inoculation with CFMMV RNA [26]. Therefore, we assume the action of an RNA-mediated resistance mechanism, as observed with other virus resistances (ref). Selections for such a resistance could be due to using very high titer.of purified virions (1 mg/ml) for inoculation. Since it is known that CP-mediated resistance is inoculum dose-dependent, and the higher the inoculum the lower the protection [19], such strong selection could discover an RNA-mediated resistant line.

In contrast to the resistant lines bearing the CP gene, all the putative 54-kDa gene bearing resistant lines exhibited a high level of resistance (immunity) to CFMMV infection, and a trace of virus could not be determined in inoculated plants by various biological and molecular methods. The selected homozygotic line I44 exhibited resistance also to graft inoculation, as did transgenic tomato bearing the CMV replicase gene [12]. The resistance to graft inoculation may indicate an inhibition of virus replication, as was characterized for transgenic tobacco protoplasts harboring the TMV 54-kDa replicase gene. The immunity of line I44 to CFMMV infection is reminiscent of immune resistance to TMV observed in transgenic tobacco transformed with the 54-kDa of TMV (REF). However *N. benthamiana* transformed with the 54-kDa gene of PMMV showed various degrees of resistance: some

transformed lines exhibited high levels of resistance (as in the case of the CFMMV 54-kDa), whereas others exhibited only a delay of virus infection ([31]).

It is clear that subgenomic RNA-1 of the 54-kDa CFMMV is expressed in CFMMV-infected cucumber (Fig. 5) as was observed previously with TMV in infected tobacco (ref quote Milt). However, to date the putative 54-kDa protein has not been detected in plants infected with a tobamovirus ([21, 34]). Therefore the importance of the expression of 54-kDa protein in transgenic plants is not clear. Resistance to PMMV was obtained with a truncated transgene of the putative 54-kDa gene of PMMV in transformed *N. benthamiana* [32]. However, Carr et al 1992 demonstrated with tobacco protoplasts that the expression of 54-kDa protein is essential for resistance against TMV infection. Additionally, the importance of transformation with the intact putative 54-kDa transgene to achieve resistance to pea early browning tobravirus in transgenic *N. benthamiana* was also demonstrated. The expression of the CFMMV 54-kDa protein in the transformed line I44 was not tested, although the transgene construct was engineered to express the putative protein. Since this putative gene product was not detected in tobamovirus-infected plants, and in addition was not detected in tobacco and *N. benthamiana* transformed with the 54-kDa of TMV and PMMV respectively, we can assume that RNA-mediated resistance occurs, rather than that the putative 54-kDa protein is involved with the resistance to CFMMV infection. However, this point needs to be evaluated more precisely.

Replicase-mediated resistance to viruses shows near immunity in many cases. However such resistance is relatively narrow spectrum, against only the parental virus and its close relatives (reference). The cucumber-infecting tobamoviruses CGMMV, KGMMV and ZGMMV are distinct viruses from CFMMV (REF); the putative 54-kDa genes of all the three show 78% homology on the RNA level and 83% identity at the protein level. However, KGMMV and ZGMMV are more closely related viruses to CFMMV than is CGMMV based on the CP amino acid sequence homology (77% and 45%) respectively (ref). The three distinct viruses (CGMMV, KGMMV and ZGMMV) break the resistance of the transgenic cucumber plants, as determined by virus accumulation and symptom expression. However, various degrees of resistance such as delay of symptom appearance and reduction in severity of symptoms were observed. Interestingly, infection of resistant line I44 with CGMMV caused mild symptoms for the experimental period of 30 days, in contrast to severe symptoms that appeared 8 dpi in the non-transformed parental cucumber. This could be due to the

reduced CGMMV accumulation in transgenic plants, although these data need to be examined further before any conclusions are drawn. Tobacco transformed with the putative 54-kDa gene of the yellow mutant of TMV showed a high level of resistance to the homologous virus, and also to the TMV U1 strain which differs by less then 1%. However, the resistance was broken by tomato mosaic virus U2-TMV and L-TMV strains which differ from TMV by ca. 20% at the nucleotide level [14].

It is likely that the mechanism of resistance of line I44 to CFMMV infection was RNA-mediated resistance, rather than protein-mediated resistance as described with PMMV (REF). Therefore, we characterized parameters associated with gene silencing mechanisms at the molecular and biological level.

a. RNA expression level of the transgene. The expression level of the 54-kDa RNA transgene in line I44 was much less than the expression of the subgenomic RNA-I of CFMMV in infected plants (Fig. 5) (the autoradiogram of the transgene was exposed 24 times longer). Interestingly, inoculation of line I44 plants with CFMMV or ZYMV did not affect the RNA expression level of the 54-kDa transgene. Similar results were observed with the expression of the 54-kDa of PMMV in transgenic resistant *N. benthamiana* [31]. However, in many studies where silencing is involved with the mechanism of virus resistance, inoculation of the transgenic resistant plants with the homologous virus reduces the detection level of the transgene RNA, due to RNA degradation as a part of the silencing mechanism (see [25]).

b. Response to infection with others viruses: pre-infection of line I44 cucumber with several potyviruses and CMV, which are known to suppress the silencing mechanism, did not break the resistance of line I44 plants to CFMMV infection (Table 4). In contrast, the resistance of *N. benthamiana* to PVA based on a silencing mechanism had been broken by infection with PVY [25].

c. Soil and graft inoculation: It was demonstrated that a signal can move from transgenic rootstock through a graft union and induce gene silencing in a scion [27]. We therefore grafted a non-transformed scion on line I44 and tested the resistance of the scion to CFMMV infection, after pre-inoculation of the rootstock with CFMMV. All the scions were infected by CFMMV and showed symptoms similar to the control (non-transformed plant) (data not shown). This result indicated that such a signal did not move via the graft or was not strong enough to induce and maintain resistance in non-transformed scion.

The accumulated data on the resistance mechanism indicate that a form of RNA-mediated resistance is involved with the resistance of line I44, however the mechanism of resistance is different from the well examined virus gene silencing phenomenon (ref- review). Additional studies will be needed to elucidate which of these proposed mechanisms effects resistance in line I44.

A practical agricultural technology is also proposed. Tobamovirus are known to be very stable, and can remain infectious for long time in dry plant tissues and as a free virion in soil and water (REF). Soil-borne virus could serve as a primary source of infection for virus epidemic development (REF). We assume that the severe CFMMV epidemic in cucumber growing in greenhouses in Israel initially came from soil containing CFMMV inoculum. Commercial grafting of cucurbits is successfully applied in the field mainly for protection of soil borne disease (ref). We therefore demonstrated for the first time protection of non-transformed cucumber from soil inoculation with CFMMV by grafting on a rootstock of homozygotic line I44 plants. The transgenic line I44 rootstock prevents movement of the virion from the soil to the non-transgenic scion. The protection could be a result of the inability of CFMMV to penetrate from the soil to the vascular parenchyma and companion cells, precluding the entrance into the phloem. Possibly tobamovirus transport is an active function and may require a limited rate of replication. Furthermore, it was reported that MP is essential for long-distance movement of TMV in tobacco phloem, which suggests that replication is required during this process [3]. An intergraft ("middle scion") harboring a transgenic 54 kDa gene of TMV [14] blocked the passage of this virus from the infected rootstock into the upper scion. In addition, transgenic tomato transformed with a defective replicase gene prevents CMV movement by graft inoculation [12]. Therefore, a transgenic rootstock can protect non-transformed scions from soil inoculation by viruses, and this technique can be used to produce non-transformed fruit, thereby reducing problems of regulation of transgenic produce.

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A novel approach to spider mite control based on expression of sarcotoxin IA peptide via virus-vector system in plants

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Abstract

Control of the spider mite, *Tetranychus cinnabarinus* is problematic, and there is a pressing need for efficient, non-hazardous and inexpensive strategies for limiting the damage it causes. The gene for the anti-bacterial peptide sarcotoxin IA of the flesh fly, *Sarcophaga peregrina* was cloned into the nonpathogenic potyvirus-based vector system ZYMV-AGII (Zucchini Yellow Mosaic Virus-AGII). Expression of sarcotoxin IA peptide via the AGII vector was detected in squash leaves infected with the AGII-sarcotoxin and it was not deleterious to the host plant. Detached leaf discs infected with the recombinant virus AGII-sarcotoxin IA were tested for spider mite control in laboratory conditions. Spider mite egg production on plants expressing the sarcotoxin IA gene was decreased by a factor of two or three compared with that on AGII-infected plants or healthy plants, respectively, while sarcotoxin IA did not show such significant effect in levels of mortality or ability to repel spider mites. Crude extract from squash leaves infected with AGII-sarcotoxin IA was found to cause a significant effect on mites' fecundity compared with extracts from AGII-treated or healthy plants. Our results demonstrate that the sarcotoxin IA effect was manifested most strongly on mite fecundity, and that this treatment shows potential for controlling spider mites in the field.

Key words: Sarcotoxin IA, Spider mite, Zucchini yellow mosaic potyvirus, cucurbit, fecundity, virus vector.

Introduction

Spider mites (Acari: tetranychidae) are major cosmopolitan pests of many crops in both developed and developing countries. The foliar pest, carmine spider mite, *Tetranychus cinnabarinus* Boisduval imposes a major constraint on cucurbit crops through its constant threat to yield quality and quantity. *T. cinnabarinus* damages cucurbit plants by piercing and sucking the foliar tissue, thus causing gradual drying of the leaves, interrupting the normal production of photosynthates and causing serious damage that leads to reduction or total loss of fruit yield (Hazen *et al.*, 1973). *T. cinnabarinus* takes about 6 days to develop from egg to adult; it can produce more than 20 generations per crop growing season (Perring and Chandler, 1996) and the female can lay as many as 200 eggs per generation, which results in population explosions. Mite control has become very problematic as currently used pesticides are rapidly losing their effectiveness because of a build-up of mite resistance (Shen, 1999). Additionally, it is difficult to attack the mites with acaricides because they locate under the leaves, the treatments harm naturally occurring mite predators, the use of pesticides is uneconomical, and toxic residues remain in the fruits (Shen, 1999).

Identifying host tolerance to *T. cinnabarinus* and other pests is often restricted to evaluating its effect on oviposition on the plant (De Ponti, 1978). Further evaluation of *T. cinnabarinus* fecundity in the laboratory suggested antibiosis as a possible mechanism of melon resistance (Edelstein *et al.*, 2003; Mansour and Karchi, 1990; Mansour *et al.*, 1994).

Sarcotoxin IA, a 62-residue peptide is one of four cecropin-type proteins encoded by the sarcotoxin I gene cluster in the flesh fly, *Sarcophaga peregrina* (Kanai and Natori, 1989). These peptides possess antibacterial activity and are important in

the immune responses of various insects (Boman *et al.*, 1987). This type of peptide, which has two amphiphilic α-helices was shown to react with the bacterial cell membrane, in which it caused a loss of potential membrane electrochemical (Nakajima *et al.*, 1987). Recently, we have expressed the sarcotoxin IA gene in *Saccharomyces cerevisiae*. The sarcotoxin-like peptide (SLP) was secreted from yeast cells and had a potent cytotoxic effect against several bacteria, including plant pathogens (Aly *et al.*, 1999). It has subsequently been shown that the sarcotoxin IA gene can be used to engineer plants for resistance to bacterial and fungal pathogens (Mitsuhara *et al.*, 2000). Moreover, it was shown that the peptide inhibited the growth of harmful bacteria in the human intestine without, however, affecting the growth of bacteria which are abundant in the intestines of healthy people (Mitsuhara *et al.*, 2001).

ZYMV–AGII (AGII) is a potyvirus-based vector system for the expression of foreign genes in cucurbits (Arazi *et al.*, 2001). The AGII virus-vector was created from an attenuated engineered ZYMV potyvirus (Gal-On and Raccah, 2000). AGII accumulates in cucurbits to the same levels as the severe ZYMV strain, without eliciting the severe phenotypic and developmental impairment caused by the wild type (Gal-On and Raccah, 2000). Indeed, infection of field-grown squash, melon and watermelon plants with AGII expressing the Bar gene conferring herbicide resistance, did not cause apparent damage or yield reduction compared with virus-free plants (Shiboleth *et al.*, 2001). In addition, the AGII viral vector was engineered to be a non-aphid transmissible (Gal-On *et al.*, 1992). This environmentally important characteristic paved the way for the biotechnological use of AGII as a vector of foreign gene expression in cucurbit fields (Shiboleth *et al.*, 2001). The aim of the present study

was to evaluate the effectiveness of sarcotoxin IA peptide, expressed via the unique AGII expression system, in controlling mites on cucurbit plants.

Materials and Methods

Insertion of Sarcotoxin IA gene into the AGII genome

The sarcotoxin IA coding sequence 186 bp, (Accession No. E01229) was PCR amplified from pFL61-SAR plasmid (Aly *et al.*, 1999) using sense primer (5'-GCTGCAGTCAAATTTCC AGAACATTTTCATTTTC -3') and antisense primer (5'-CAGTGTCGAC ACCTCTGGCTGTAGCAGC-3'). The restriction enzymes PstI and SalI sites (underlined) (Fig. 1A) were inserted to the ends of the 5' and 3' of the sarcotoxin IA cDNA. The amplified fragments were double-digested by the respective enzymes and cloned into the appropriate sites within the polylinker of the AGII genome between the coat protein (CP) and the NIb coding regions (Arazi *et al.*, 2001), to create AGII-sarcotoxin (AGII-Sarco.) as shown in Figure 1A.

Plant growth conditions and virus inoculation

Squash (*Cucurbita pepo* L. cv. Ma'ayan) plants were grown in a temperature-controlled greenhouse at 25°C with a photoperiod of 14 h, or in controlled growth chamber at the required temperature (20-35°C). Fully expanded cotyledons of each plant were inoculated by particle bombardment with a hand held device (HandGun) as described by (Gal-On *et al.*, 1997).

RT-PCR analysis of recombinant virus progeny

Expression of the viral RNA was determined by RT-PCR analysis of total RNA extracted from seedlings 10 days after germination. Young leaf tissue (second leaf from the top) (100 mg) was ground to a fine powder in liquid nitrogen and total RNA was extracted with the TRI-REAGENT kit (Molecular Research Center, Inc., Cincinnati, OH, USA), according to the manufacturer's instructions. RT-PCR of the viral progeny was conducted by a modified one-tube, single-step method (Sellner *et al.*, 1992) as described in (Arazi *et al.*, 2001). The reaction was performed with the ZYMV-AGII polylinker flanking primers: forward from the NIb gene 5'-AGCTCCATACATAGCTGA GACA-3' and reverse primer from the CP gene 5'-TGGTTGAACCAAGAGGCGAA-3' (Figure 1).

Expression of sarcotoxin IA peptide in squash leaves

The expression of sarcotoxin IA peptide in squash leaves via the AGII vector was characterized by Western blot analysis. Total proteins from 100 mg of squash leaves 20 days post infection with AGII-Sarco. or AGII, or from healthy plants were extracted and applied to protein tricin-SDS gel and blotted with anti-sarcotoxin IA antibody at a dilution of 1:1000, according to (Mitsuhara *et al.*, 2000).

Mite stock culture

The spider mites were originally reared on 2- to 3-week-old kidney bean *Phaseolus vulgaris* L. (Fasaceae) plants in 25×32×8-cm pots which were seeded at 7-day intervals. Then the mites were transferred to squash plants for one month for adaptation. Squash plants with *T. cinnabarinus* mites were reared in a controlled-

climate room at 25-27^{0}C, 60±5% RH and 16-h photoperiod, with fluorescent lamps with a light intensity of ca. 2000 lux.

Mites bioassay on virus-inoculated plants

Five discs (22 mm diameter) were punched out from the third leaf of three squash plants that had been inoculated either with AGII or AGII-Sarco., or had not been inoculated (healthy plants), and were placed abaxial surface upwards on filter papers in 90-mm-diameter Petri dishes. Ten adult female mites, 3-5 days old, were transferred from the colony to each disc. Each paper with its discs was placed on a sheet of foam floating on water in the Petri dish. The dishes with the mites were kept in a controlled-climate room at 25 ± 1°C (Mansour *et al.*, 1994). The numbers of dead mites and newly laid eggs were counted three days after inoculation. Each experiment had a completely randomized design with three separate experiments with five replicates for each treatment.

Mite bioassay with plant extract

Samples (10 g) of fresh squash leaf, from plants inoculated with AGII or AGII-Sarco., or from healthy plants, were homogenized in 100 ml of double-distilled water in a Waring blender. The crude extract was then filtered through a fiber-glass filter and centrifuged at 5000 g for 5 min to remove debris. The bioassay was conducted according to Mansour (1994). Kidney bean *Phaseolus vulgaris* L. (Fasaceae) plant discs (22 mm in diameter) were immediately immersed in the filtrate extract for 5 s, and dried on Whatman paper placed on a sheet of plastic foam for 1 h. Ten female

mites were placed on each leaf disc and tested for the presence of an ingredient bioactive against *T. cinnabarinus*.

Statistical analysis

The data represent the results of three separate experiments. Averages were separated by using the Tukey-kramer Honestly Significant Differences (HSD) test at $p \leq 0.05$. There was no treatment interaction between any of the variables; therefore, the results of the separate experiments were combined and are presented as five replications for each treatment.

Results

Verification of progeny virus and sarcotoxin IA expression in plant

The sarcotoxin-IA cDNA gene (189 bp) was inserted between the NIb (replicase) and CP (coat protein) genes of the AGII virus vector by use of a polylinker-cloning site between the NIa proteinase cleavage sites, as shown in Figure 1A (Arazi *et al.*, 2001; Shiboleth *et al.*, 2001). The inserted sarcotoxin IA gene was designed to create an in-frame translational fusion of the recombinant virus AGII-sarco. Proteolysis of the nascent AGII-Sarco. polyprotein by NIa protease in trans was predicted to yield recombinant sarcotoxin protein with a single additional amino acid residue at the N-terminal (S) and seven amino acid residues (VDTVMLQ) at the C-terminus (Figure 1A). The presence of the intact sarcotoxin coding sequences in the recombinant virus was verified by RT-PCR of the viral progeny 10 days post inoculation (Figure 1B). To confirm the existence of the sarcotoxin IA gene within the AGII viral progeny, the RT-PCR product was digested with *Mlu* I restriction enzyme. The three cDNA fragments

obtained by *Mlu*I digestion of AGII-Sarco. cDNA compared to the two fragments with the AGII alone confirmed the existence of the sarcotoxin IA gene (Figure 1B). In addition, direct sequencing of the amplified RT-PCR cDNA confirmed the presence of the intact sarcotoxin 1A in the virus genome (data not shown). Western blot analysis confirmed the presence of sarcotoxin IA peptide (8 kDa) only in squash leaves infected by AGII-Sarco. (Figure 1C, lane 3). In addition, squash plants expressing the sarcotoxin IA gene showed normal growth similar to that of virus-free plants which indicates that the sarcotoxin IA peptide had no effect on plant developmental (Figure 4A).

Effect of squash plants expressing sarcotoxin IA in controlling spider mites

To evaluate the effect of sarcotoxin IA-expressing plants on the spider mite (*T. cinnabarinus*), three biological parameters were measured: fecundity, repellency and mortality of *T. cinnabarinus*. The numbers of dead mites, repelled mites and newly laid eggs were counted 3 days after mites were placed on leaf discs taken from squash plants inoculated with AGII-Sarco. or with AGII, or from virus-free control plants (healthy). The total numbers of eggs laid on leaf discs were 19 in squash inoculated with AGII-Sarco., and 41 and 59 in AGII-infected control plants and healthy plants respectively (Figure 2A). These results showed a significant reduction in the total number of eggs laid on leaf discs expressing the sarcotoxin IA peptide as compared with that on the controls. No such significant differences in mortality (Figure 2B) and repellency (data not shown) were found between plants expressing the AGII-Sarco. and the controls expressing AGII alone.

Effect of extract from squash plants expressing sarcotoxin IA in controlling spider mites

Indirect effects of sarcotoxin IA on *T. cinnabarinus* spider mite mortality, fecundity and repellency was tested. Experiments were conducted with kidney bean *Phaseolus vulgaris* L. (Fasaceae) leaf discs (the preferred host for spider mites) treated with crude extract from plants that had been inoculated with AGII-Sarco. or AGII virus, or from healthy plants. Mites ingested onto kidney bean leaf discs treated with AGII-Sarco. oviposited significantly fewer eggs (83) than those ingested onto discs treated with AGII and healthy plant extract (134 and 132, respectively) (Figure 3A). Mortality was significantly greater among mites feeding on AGII-Sarco. extract than among those on control extracts from AGII and virus-free plants (Figure 3B). In addition, AGII-Sarco. extract increased the repellency of mites, compared with that of the control AGII-treated disks but the difference was not significant (Figure 3C).

Discussion

In this study we tested the antibacterial peptide sarcotoxin IA as a new means for spider mite control. Expression of the recombinant peptide by a non-pathogenic potyvirus, AGII was confirmed by RT-PCR and Western blot analysis (Figure 1). The immunoreactive signal detected around 8 kDa. in AGII-Sarco. treatment was most probably a dimer of the synthetic sarcotoxin IA, since the expected size of the monomer is around 4.2 kDa (Mitsuhara *et al.*, 2000). We have previously shown (Arazi *et al.*, 2001) that the foreign genes from plants (MAP30 and GAP31) (Arazi *et al.*, 2002), bacteria (*bar*) (Shiboleth *et al.*, 2001) and humans (interferon alpha 2a) (Arazi *et al.*, 2001), expressed via AGII, were functional with the addition of seven

amino acids at the C terminal. Since the parental AGII virus did not affect virus accumulation and plant development, we considered that the expressed sarcotoxin 1A gene with the extra amino acids, like other foreign genes (Arazi *et al.*, 2002; Arazi *et al.*, 2001; Shiboleth *et al.*, 2001), could maintain its bactericidal activity and would not interfere with the AGII virus life cycle, i.e., movement or replication. Squash plants expressing sarcotoxin IA grew and developed normally and were not altered by the expression of this peptide. A similar lack of effects on plant development was also reported in transgenic tobacco plants expressing sarcotoxin IA (Mitsuhara *et al.*, 2000).

To evaluate spider mite control by the recombinant peptide expressed in squash plants, three biological parameters were tested: fecundity, mortality and repellency. For these tests two sets of experiments were conducted: first, direct feeding of *T. cinnabarinus* spider mites on leaf discs expressing sarcotoxin IA; and, second, indirect feeding of spider mites on bean leaf discs treated with sarcotoxin IA extract.

The results obtained from direct feeding on squash (Figure 2A) showed that AGII-Sarco. treatment was effective on mite fecundity in that it significantly reduced the total number of eggs laid on leaf discs expressing the sarcotoxin IA peptide, compared with the number on the control plants. In contrast, no such significant differences in mortality (Figure 2B) and repellency (data not shown) were found between plants expressing the AGII-Sarco. and those expressing AGII alone. In indirect application, when leaf disks from the mites' favorite host, the kidney bean, were coated with squash extracts (from plants treated with AGII-Sarco. or AGII, or from virus-free plants) a significant effect was observed on the number of eggs laid. With the same indirect application mortality was significantly greater in mites ingested onto AGII-Sarco. extract than in those on control extract plant (Figure 3B). However, although,

AGII-Sarco. increased repellency to the mites, the effect obtained was not significant (Figure 3C). The evidence that the fecundity of the mites was reduced by both feeding procedures reinforces the finding that the recombinant sarcotoxin IA affects mite egg production. However, the observed difference between the direct and indirect feeding systems, in their effects on mortality might indicate that the mites' response to sarcotoxin IA accumulation is dosage dependent. In the direct application system the expression level of sarcotoxin IA was not equal in all plant cells, as demonstrated by the distribution of expression of the reporter gene GUS in the same system (Figure 4B). The uncolored area between the blue areas (Figure 4B) indicates that GUS was not expressed, which could be because the virus was not present in the cells, or that it was present but did not replicate. The mosaic pattern of expression of reporter genes is a well known phenomenon with potyvirus vector systems (Dolja *et al.*, 1992) Thus, in the present study, the indirect application by treating leaf discs with sarcotoxin IA extract would have covered the leaf more uniformly and with more concentrated recombinant sarcotoxin IA, so that this mode of application would have a more significant effect on mite mortality than the direct mode.

On the other hand, it is likely that the sarcotoxin IA peptide has no effect on repellency, since its biological function is not related to repulsion.

Nevertheless, we observed significant differences in fecundity between plants expressing AGII-sarc or AGII alone. The fecundity findings were consistent with the data obtained from both direct (detached leaf discs) and indirect (plant extract) feeding assays of *T. cinnabarinus*.

Breeuwer *et al.* (1996) reported that intracellular microorganisms such as Wolbachia co-existed in spider mites and altered reproduction in arthropods. Deleine et al., (2001)

showed that removal of the intracellular bacteria (Wolbachia) from parasitic wasps by means of antibiotics inhibited oogenesis, which prevented the wasps from reproducing . Meltzer (1972) showed that fecundity and growth of newly hatched larvae of *T. cinnabarinus* were severely affected by exposure to the antibiotic MYC 8005 . Accordingly, we postulate that the significant reduction in the total number of eggs obtained on leaf disks expressing the sarcotoxin peptide could be due to the effect of sarcotoxin as an antibacterial peptide on intracellular microorganisms that coexist in spider mites and that this, in turn, altered the reproduction of the mites. Therefore, sarcotoxin (as an antibacterial peptide) could possibly provide a safe means for eliciting crop resistance against spider mites.

Further research is needed, to test the direct effect of sarcotoxin IA peptide on *T. cinnabarinus* spider mites and to characterize specific microorganisms that coexist with spider mite. Since the AGII vector is environmentally safe, does not impair the growth of the cucurbit host, cannot be transmitted between plants by aphids (the natural vector), is not transmitted via seeds, and is biodegradable in the soil, it could be potentially used as a bio-control application against mites.

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Figure 1. Expression of *sarcotoxin IA gene* via ZYMV-AGII. (A) Schematic representation of the AGII genome shows non-coding (shaded) and coding (open) regions, and the inserted foreign gene sarcotoxin IA (Sarco.). Arrows indicate NIa protease involved in proteolysis of the foreign gene product. NIa cleavage sites are indicated by /. Amino acid sequences corresponding to the NIa protease recognition motif are indicated in bold. (B) Analysis by RT-PCR of AGII-Sarco. viral progeny RNAs. Total RNA was extracted from leaves of squash systemically infected with AGII-Sarco. or AGII, or from virus-free plants, 18 days post inoculation, and subjected to RT-PCR with primers flanking the foreign gene insertion point. The arrows below show positions of RT-PCR primers relative to AGII genome schematically. The expected sizes (bp) of the fragment with and without Sarco. are 665 and 476 bp, respectively. (C) Western blot of squash leaf discs expressing sarcotoxin IA. Total proteins were extracted from (100 mg) squash leaf discs inoculated with AGII or AGII-Sarco., 20 days post infection, and subjected to protein gel blotting with anti-sarcotoxin IA antibody. The Sarco. lane is a positive control (40 ng of synthetic sarcotoxin IA peptide); arrows indicate the monomer (4.2 kDa) and the dimer size (8.2 kDa) of sarcotoxin IA peptide. The right-hand arrow indicates a specific band that reacted with the sarcotoxin IA antibody in AGII-Sarco. lane.

Figure 2. Effect of sarcotoxin IA peptide expressed via AGII in squash leaf discs, on fecundity (A) and mortality (B) of *T. cinnabarinus.* Five discs from squash plants inoculated with AGII or AGII-Sarco., or from healthy plants were inoculated with 10 *T. cinnabarinus* females per disc (total of 50 females per treatment) and antibiosis was

evaluated 3 days after inoculation. Column values represent the means of five replications, with the standard error. Means within a column followed by the same letter are not significantly different according to the Tukey-Kramer test at $p \leq 0.05$.

Figure 3. Effect of total crude extract from squash leaf discs expressing AGII or AGII-Sarco., or from control discs, on: (A) fecundity of; (B) mortality of; and (C) repellency against *T. cinnabarinus*. Five bean leaf discs were immersed for 5 s in extracts from AGII- or AGII-Sarco.-infected, or healthy plants, and *T. cinnabarinus* antibiosis was assayed as described in Material and Methods. A: Numbers of *T. cinnabarinus* eggs laid on bean leaf discs 3 days after treatment. B: Numbers of dead mites on bean leaf discs 3 days after treatment. C: Numbers of repelled mites 3 days after treatment. Column values represent the means of five replications with the standard error. Means within a column followed by the same letter are not significantly different according to the Tukey-Kramer test at $p \leq 0.05$.

Figure 4. A. Squash leaves infected by AGII-Sarco. and AGII 18 days post inoculation. Also shown is a virus-free (mock-inoculated) leaf. All plants were inoculated at the same age at the same time, and the 4th leaf from the apex of each plant was photographed at the same time. B. Cucumber leaf infected with AG-GUS, 18 dpi. GUS expression was visualized by washing in x-gluc and then twice in 70% EtOH over a 24-h period until all chlorophyll was removed.

What is claimed:

1. An improved plant having distinguishing characteristics essentially as described herein or depicted in the figures.

2. A method of producing an improved plant having distinguishing characteristics essentially as described herein or depicted in the figures.

3. Agricultural produce derived from an improved plant having distinguishing characteristics essentially as described herein or depicted in the figures.

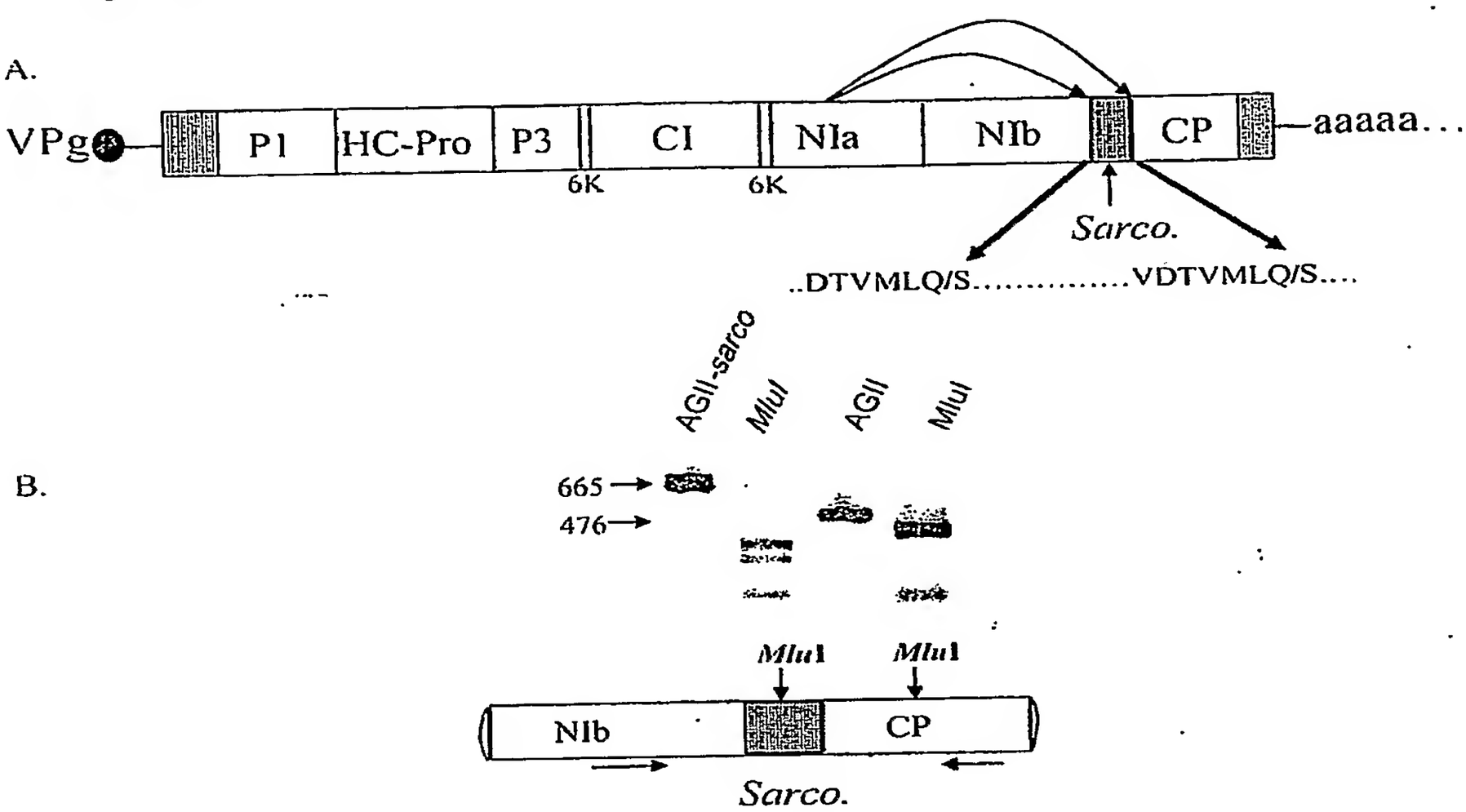
4. A method for pest control having distinguishing characteristics essentially as described herein or depicted in the figures.

5. A method for use of an antibacterial peptide having distinguishing characteristics essentially as described herein or depicted in the figures.

6. The method of claim 5, wherein said antibacterial peptide includes sarcotoxin IA of the flesh fly, *Sarcophaga peregrina* or a functional portion thereof.

Figure 1.

A.



B.

C.

Figure 2.

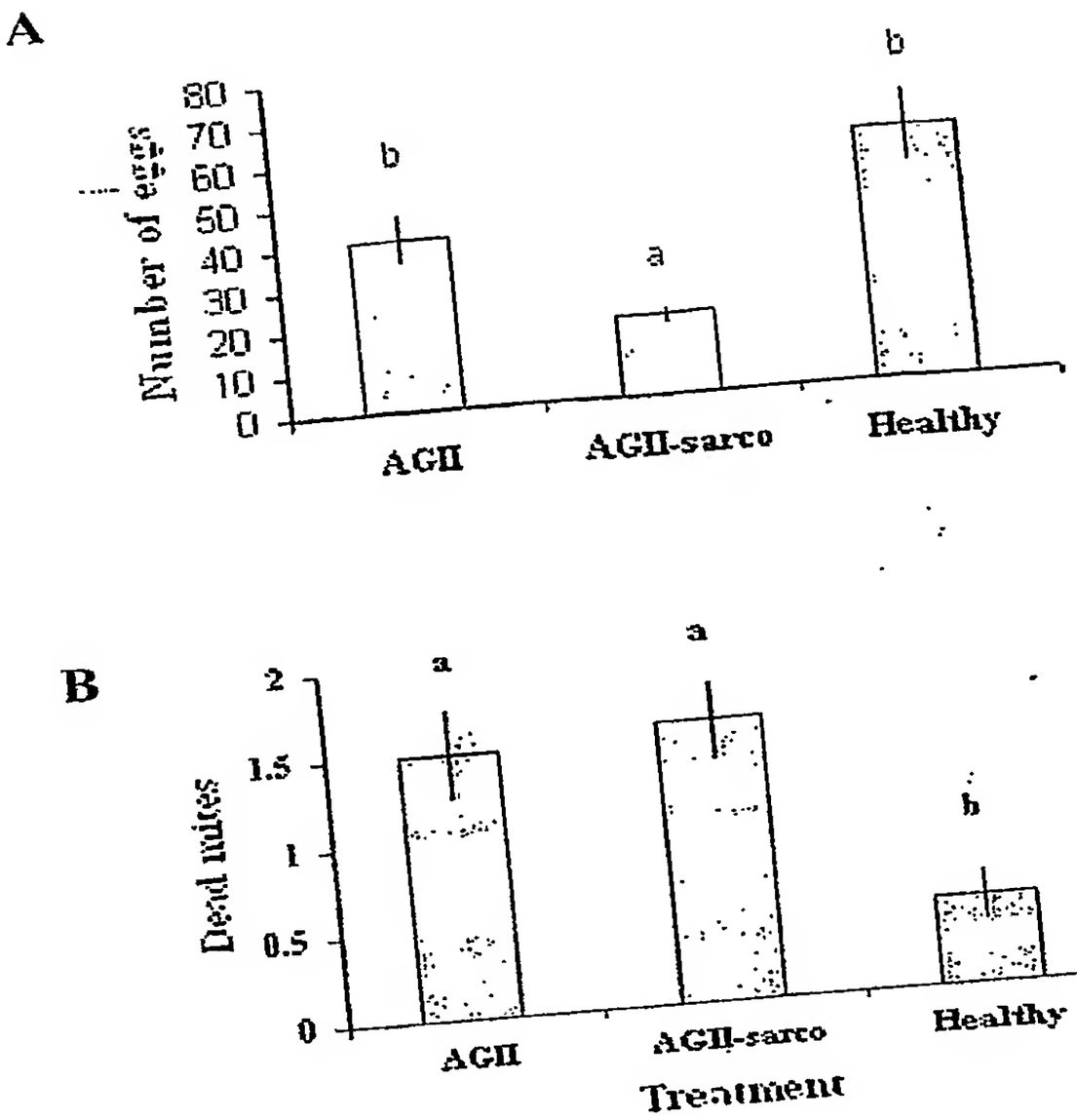
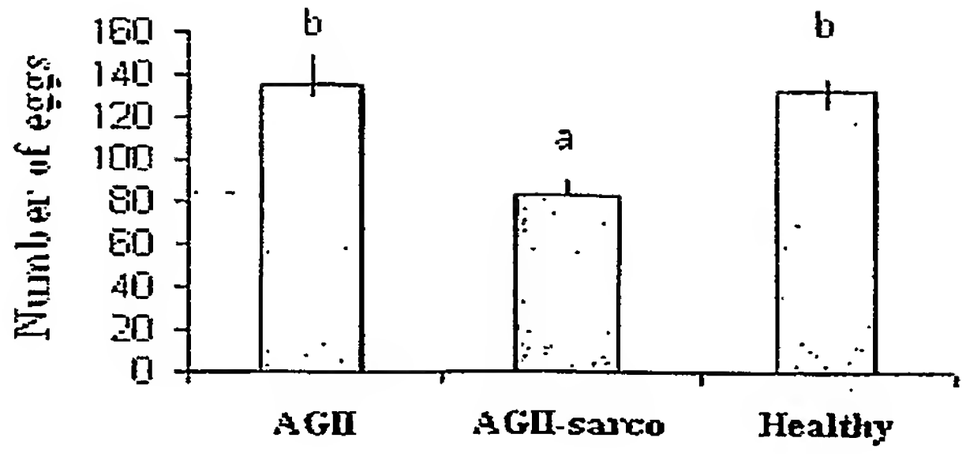


Figure 3.

A



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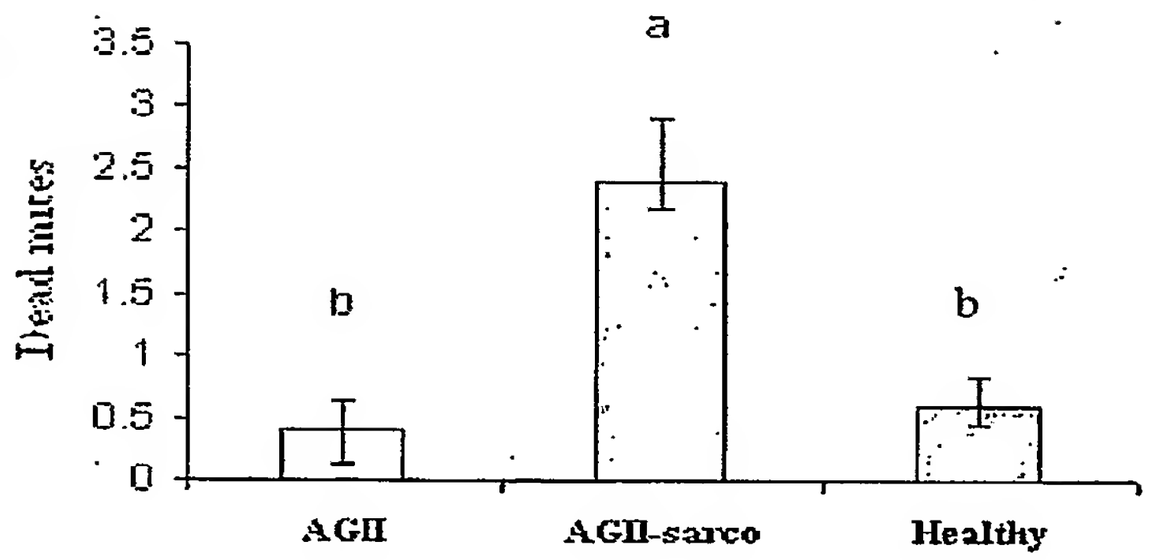

C

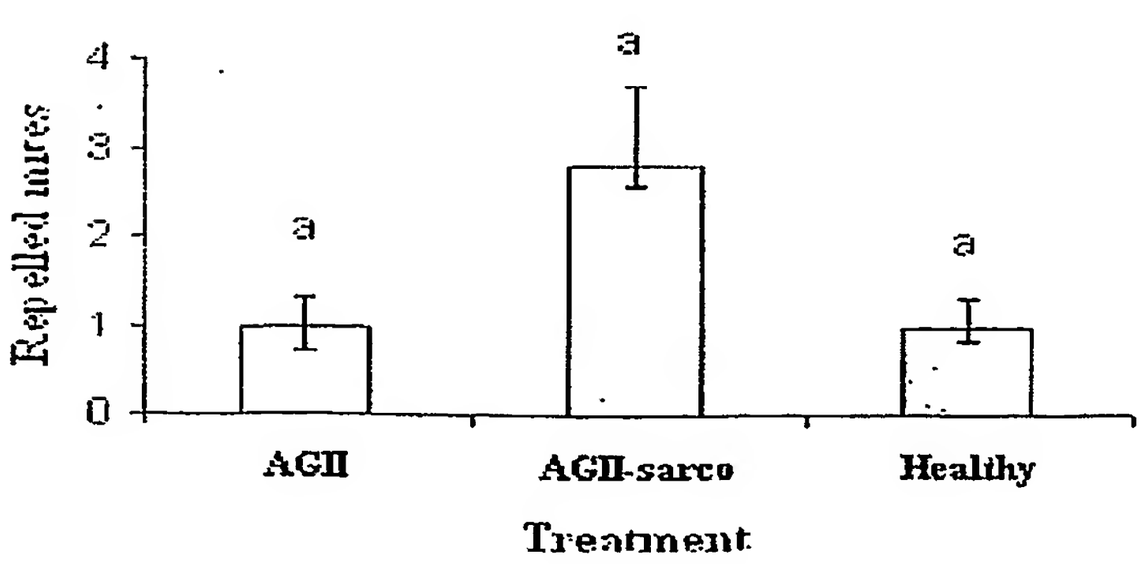

Figure 4.